The Ohio State University

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Senior Thesis Defense for Distinction in Research in Biology

“Effects of Jack Bean urease on Insect Blood”

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Abstract

Jackbean (*Canavalia ensiformis*) ureases are entomotoxic to hemipteran insects, an effect partially due to the release of internal peptides by insect's cathepsin- like digestive enzymes. Here we studied the effects of the major jackbean urease isoform (JBU) on isolated hemocytes of 5th instars *Dysdercus peruvianus'*. JBU (200 nM) was found to trigger *in vitro* microaggregation of hemocytes, visualized under light microscopy and also after DAPI stain. Hemocytes were suspended in a calcium free saline before exposure to JBU, and the absence of external calcium did not change the aggregating effect of JBU. In the presence of JBU, there were a smaller number of melanized cells in the calcium-free saline than in regular saline. The eicosanoid synthesis indirect inhibitor dexamethasone was used for testing a possible eicosanoid modulated aggregation. Dexamethasone (50 μM) was found to decrease the aggregated ion of hemocytes caused by JBU. The results suggest that JBU is activating immune reactions in *D. peruvianus* isolated hemocytes, probably through eicosanoid metabolites, as was seen with mammal cellular models.

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1. Introduction and Objectives

1.1. Ureases

The legume *Canavalia ensiformis*, popularly known as 'Jackbean', is highly resistant to insects and its seeds are rich sources of proteins such as ureases (Sumner 1926; Carlini and Polacco, 2008). Ureases are nickel-dependent metalloenzymes that catalyze urea hydrolysis into two molecules of ammonia and one molecule of carbon dioxide (Dixon *et al*., 1975). These proteins can be found in plants, fungi and bacteria but are not synthesized by animals (Mobley and Hausinger, 1989). Previous studies have shown that *jackbean* ureases are lethal when fed to insects and show persistence of their insecticidal property after treatment with an irreversible urease inhibitor, demonstrating that a protein domain distinct from the active site is involved in entomotoxic activity (Carlini *et al.,* 1997; Follmer *et al*., 2004). This toxicity is partially dependent on urease hydrolysis inside the insect's midgut by cathepsin B and D digestive enzymes, which cleave the molecule in specific sites for the release of peptides with toxic activity (Piovesan *et al*., 2008; Defferrari *et al*., 2011). After *in vitro* hydrolysis of a urease isoform from Jackbean, called canatoxin (cntx), by the coleopteran *Callosobruchus maculatus'* digestive enzymes, a 10 kD peptide was isolated. That fragment was highly toxic when administered orally to insects of different orders. Based on the sequence of this peptide, a recombinant peptide was designed, Jaburetox-2Ec that is also toxic to all insects tested so far (Ferreira-DaSilva *et al*., 2000).

The physiological role of urease in plants may have been protective against pathogens and phytophagous insects. Ureases were detected in various plants by testing immunoreactivity with polyclonal anti-canatoxin antibodies, suggesting a process of evolutionary conservation of antigenic determinants (Carlini *et al.*, 1991). In addition, reports that the content of urease increases progressively during seed maturation reinforce the idea that these proteins play an important role in plant defense (Barcellos *et al*., 1993). Also, tests demonstrate the effects of the intact JBU on the hemipteran model *Rhodnius prolixus*, where it was observed that JBU potentiates serotonin-stimulated contractions of the anterior midgut and that one cyclooxygenase inhibitor is capable of blocking this effect. In the same study it was demonstrated that cyclooxygenase products (prostaglandins) increase inside the midgut after JBU treatment, suggesting eicosanoids might be playing a role as second messengers on this effect (Stanisçuaski *et al*., 2010). Ureases are able to activate different cells through eicosanoid pathways; for example, *H. pylori* urease induces platelet activation via lipoxygenase and

subsequent release of hydroperoxides (Wassermann *et al*., 2010) and as cntx that activates the production of eicosanoids and pathways of lipoxygenase, inducing exocytosis and changes in levels and flows of intracellular Ca^{2+} in different mammalian cells (Carlini *et al*., 1985; Barja-Fidalgo *et al.*, 1991a and 1991b; Ghazaleh *et al.,* 1992 and 1997).

1.2. Eicosanoids and immune reactions in insects

Eicosanoids are synthesized from fatty acids, mainly from arachidonic acid (AA) (20: 4n–6) released upon cell stimulation from membrane phospholipids, via activation of a phospholipase A2 (PLA2) (Fig. 1). AA then follows three possible oxygenation pathways, cyclooxygenase (COX - yielding prostaglandins), cytochrome P450-expoxygenase and lipoxygenase (LOX). Most of the work done with insects focuses on the products of COX, especially the prostaglandins (PGs). PGs and other eicosanoids serve as central mediators within insect immunity (Stanley, 2000; Stanely *et al.*, 2009), and were detected in reproductive tissues of crickets (Loher *et al*., 1981), in the gut of caterpillars (Buyukguzel *et al*., 2002) and in tissues of the immune system, like hemocytes (Gadelhak *et al*., 1995) and fat body (Stanley-Samuelson and Ogg, 1994; Tunaz *et al*., 2001). Cellular reactions are coordinated by eicosanoids and are involved in several defense functions, such as phagocytosis, microaggregation, nodulation, cell-spreading, encapsulation, and hemocyte migration. Insect immunity includes at least 3 types of cellular reactions to invasions: phagocytosis, nodulation and encapsulation. Microaggregation is a part of the nodulation process and clears bacterial infections from the hemocoel. Nodulation begins with the entrapment of bacterial cells that form microaggregates that grow by joining with additional cells to form nodules. The nodulation process is finished when layers of phagocytes are attached to mature nodules. The melanization process leaves observable nodules connected to the inner sides of the body or wall of various organs (Stanely *et al*., 2009).

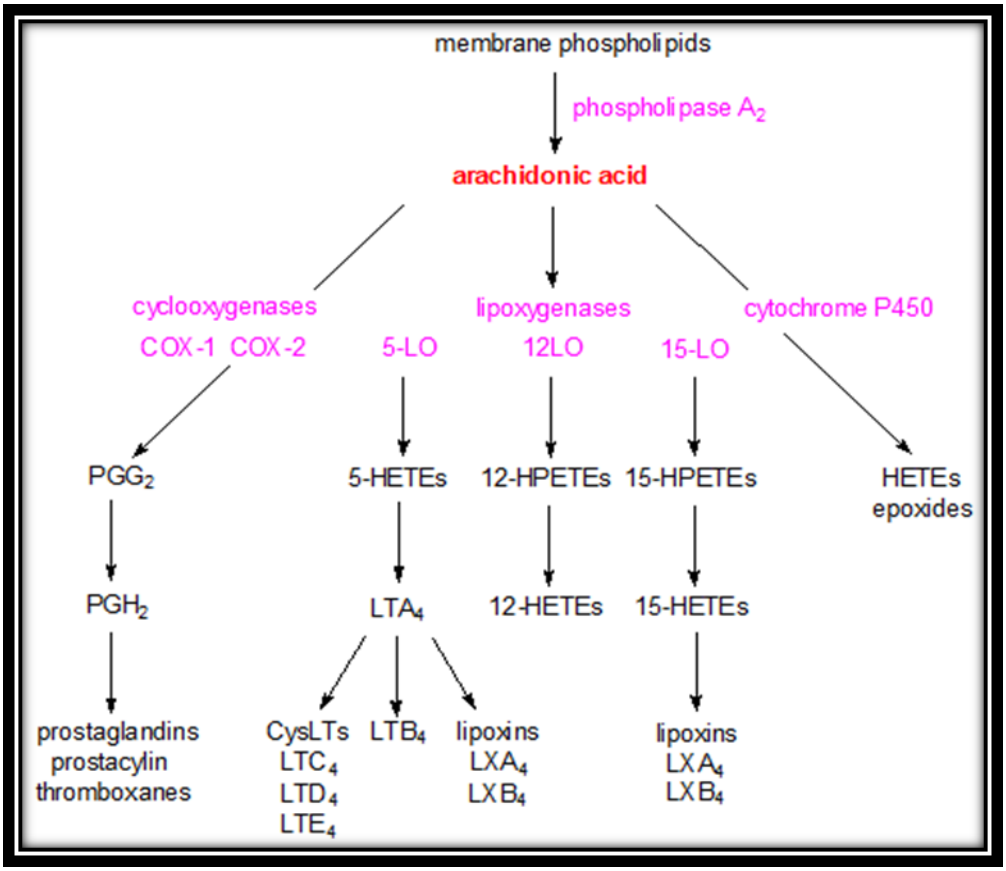

Figure 1. Eicosanoids biosynthesis scheme (Stanley *et al*., 2009).

PLA_2 - phospholipase A2

LOX- lipoxygenase

COX-cyclooxygenase

1.3. *Dysdercus peruvianus* and hemocyte classification

Insect hemocytes include the following types: (1) plasmatocytes, (2) granular hemocytes or granulocytes, (3) oenocytes, (4) prohemocytes, (5) spherule cells or spherulocytes (Lavine and Strand, 2002; Price and Ratcliffe, 2004; Costa *et al*., 2005; Ribeiro and Brehelin, 2006; Giglio *et al.*, 2008; Strand, 2008). Plasmatocytes are common hemocytes and have pseudopods resembling polymorphic amoebocytes. Granulocytes show scattered cytoplasmic granules that resemble plasmatocytes. Oenocytes are oval and elongated in shape with a smaller nucleus than the others. Prohemocytes are small, round cells that are normally found undergoing mitotic division. Spherulocytes, or adipohemocytes, are large hemocytes that have the appearance of circulating fat bodies with small nuclei (Barracco *et al.*, 1987). Not all species have all types, and the relative proportions of each vary across insect groups and species.

Here, we were able to study the effects of vitality and quantity of hemocytes after JBU treatment. Our model of study was the phytophagous hemipteran *Dysdercus peruvianus*, also known as the “cotton stainer bug”. *D. peruvianus* feeds on cotton seeds, harming them and also staining the cotton fibers; it is an imported pest species that not only minimizes cotton production, but also is a vector for phytopathogenic microorganisms (Gallo *et al*., 1988). Nymphs have been found to be susceptible to urease isoforms, while adults were not (Stanisçuaski *et al*., 2005; Piovesan *et al*., 2008). Therefore, we used 5th instars as our subjects of study.

Studies of biosynthetic routes can have implication for human health. In mammals, including humans, the glucocorticoid dexamethasone acts by increasing the expression of annexin A1, a protein that binds to and inhibits phospholipase A2 (PLA2) eicosanoid production by blocking the activity of PLA2 (Herbert *et al*., 2007). Annexin A1 is active in various aspects of cell biology (Lim and Pervaiz, 2007). These inhibitors, like Dex, are generally used to alleviate pain or inflammation. Dex’s effects have been found to be reversed within insects treated with arachidonic acid (AA). Therefore, it can be inferred that the actions of Dex in insects are linked to the biosynthesis of eicosanoids (Stanley *et al*., 2000). From the previous evidence of effects caused by urease that produced eicosanoid reactions in both mammalian models and *Rhodnius prolixus*, our objective is to study the activity of *Canavalia ensiformis* urease on the hemocytes of *Dysdercus peruvianus*. The immune system of insects has been found to be centrally mediated by eicosanoids, where cellular reactions are the first response. This is an interesting target for the understanding of the mechanism of action of JBU on insects. This potential finding

could also open doors to new pharmaceutical methods in the treatments of infections that deflect the mammalian immune system.

2. Materials and Methods

2.1. Reagents

Canavalia ensiformis urease (JBU), dexamethasone and Trypan blue were obtained from Sigma Chemical Company (Saint Louis, USA). DAPI (4,6–Diamidine-2-phenylindole dihydrochloride) was obtained from Roche Diagnostics GmbH (Mannheim, Germany).

2.2. Insects

A colony of *D. peruvianus*, has been maintained in the laboratory for eleven years, as described elsewhere (Stanisçuaski *et al*., 2005). The insects develop from eggs through five nymphal stages before metamorphosing to adults, in about 20–25 days. The insects were fed with cotton seeds (*Gossipium hirsutum*) and had free access to water. 5^{th} instars were used in all experiments.

2.3. Hemolymph collection and hemocytes isolation

D. peruvianus hemolymph samples were collected carefully from a cut leg with a 20-µl micropipette, as described by Machado *et al*. (2006), with some modifications. The samples consisted of a total of 8 bugs and approximately 4 µl of hemolymph per bug. The collections were immediately added to ice cold anticoagulant solution (62 mM sodium chloride, 10 mM ethylene diamine tetra-acetic acid, 26 mM citric acid, 100 mM glucose, pH 4.6) at a proportion of 1:5 (anticoagulant: hemolymph). Hemolymph was then centrifuged at 4°C, 4,400 *g,* for 2 min and the pelleted hemocytes were washed two times and then re-suspended in insect saline, prepared as described by Meredith *et al*.(1984) with modifications: 20 mM NaCl, 24 mM KCl, 2 mM $CaCl_2$, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 6.7 mM glucose, pH 6.9, and 20 mM NaCl, 24 mM

KCl, 4 mM $NaHCO_3$, 2 mM $MgCl_2$, 6.7 mM glucose, pH 6.9 for the calcium free saline. The volume of saline for hemocytes re-suspension was the same as the initial hemolymph volume.

2.4. Microaggregation assays

Hemocytes *in vitro* microaggregation was assayed following the method of Miller and Stanley (2001) as adapted by Garcia *et al.* (2004). After re-suspension in saline, cells were treated with JBU (200 nM) and incubated for 1 hour at room temperature. In order to analyze the involvement of eicosanoids, cells were incubated at room temperature for 30 minutes with dexamethasone in different concentrations prior to addition of JBU. Three control groups were used: (1) cells were re-suspended in saline and incubated for 1 hour at room temperature, (2) cells were re-suspended in saline and treated exclusively with dexamethasone (0.05 mM) for 1.5 hour, room temperature, and (3) cells were re-suspended in saline and incubated in presence of ethanol for 1.5 hour – this serving as a control for the dexamethasone experiments, since the reagent was diluted in ethanol. After the cells were submitted to treatments, DAPI (0.03 µM) was used to fluorescently stain viable cells, and was incubated for 30 minutes at room temperature. Alternatively, Trypan blue (1:10) was added to DAPI untreated cells prior to microscopic analysis in order to differentiate damaged from intact hemocytes (where unstained cells are viable and blue cells are in lysis process). The samples were analyzed in a Neubauer chamber under optical and/or epifluorescent microscopy. The microscope was an Axioskop 40 from Zeiss (Germany), and the magnification used was 20x/ 0.5 for all pictures.

3. Results

3.1. Hemocytes microaggregation induced by JBU

In vitro hemocyte microaggregate formation may be influenced by various factors, including the handling during preparations (Garcia *et al*., 2004). Here we observed that after one hour incubation at room temperature, JBU (200 nM) induced hemocyte microaggregation (Fig. 2B and Table 1). Control hemocytes, which were collected and kept in the same conditions as JBU treatments, were incubated in saline alone and did not produce microaggregates (Fig. 2A), indicating that the handling and the protocol were adequate. Besides microaggregate formation, it was possible to detect the presence of melanized cells and larger microaggregates, suggesting that JBU is not only inducing cellular reactions but also leading to a humeral response (Fig. 3A)

JBU-induced microaggregation was tested in the presence of a PLA2 indirect inhibitor, glucocorticoid dexamethasone, which apparently inhibited aggregation (Fig. 4), suggesting that the effect might be mediated by eicosanoids. The hemocytes were not affected and did not aggregate when tested only with dexamethasone or ethanol, in the absence of JBU (data not shown). The importance of calcium on JBU activity was seen in a previous study, where extracellular calcium was found to be necessary to JBU's effect on *Rhodnius prolixus* diuresis inhibition (Stanisçuaski *et al*., 2009). We also tested JBU effect on hemocytes in calcium-free saline, which did not change JBU aggregative activity completely, but reduced the melanized cells and aggregates (Fig. 3B and Table 1).

Table 1. Counts of hemocytes on a Neubauer chamber under optical microscopy with 20x/0.5 magnification. Hemocytes were incubated in regular saline (control), JBU (200 nM) in regular saline or JBU (200 nM) in calcium free saline.

Treatment	**Number of cells per mL of hemolymph**	**Number of microaggregates per mL of hemolymph**
Regular saline (control)	67 $x10^{+4}$	0
Regular saline + JBU	57 $x10^{+4}$	19 $x10^{+4}$
Calcium free saline + JBU	57 $x10^{+4}$	15 $x10^{+4}$

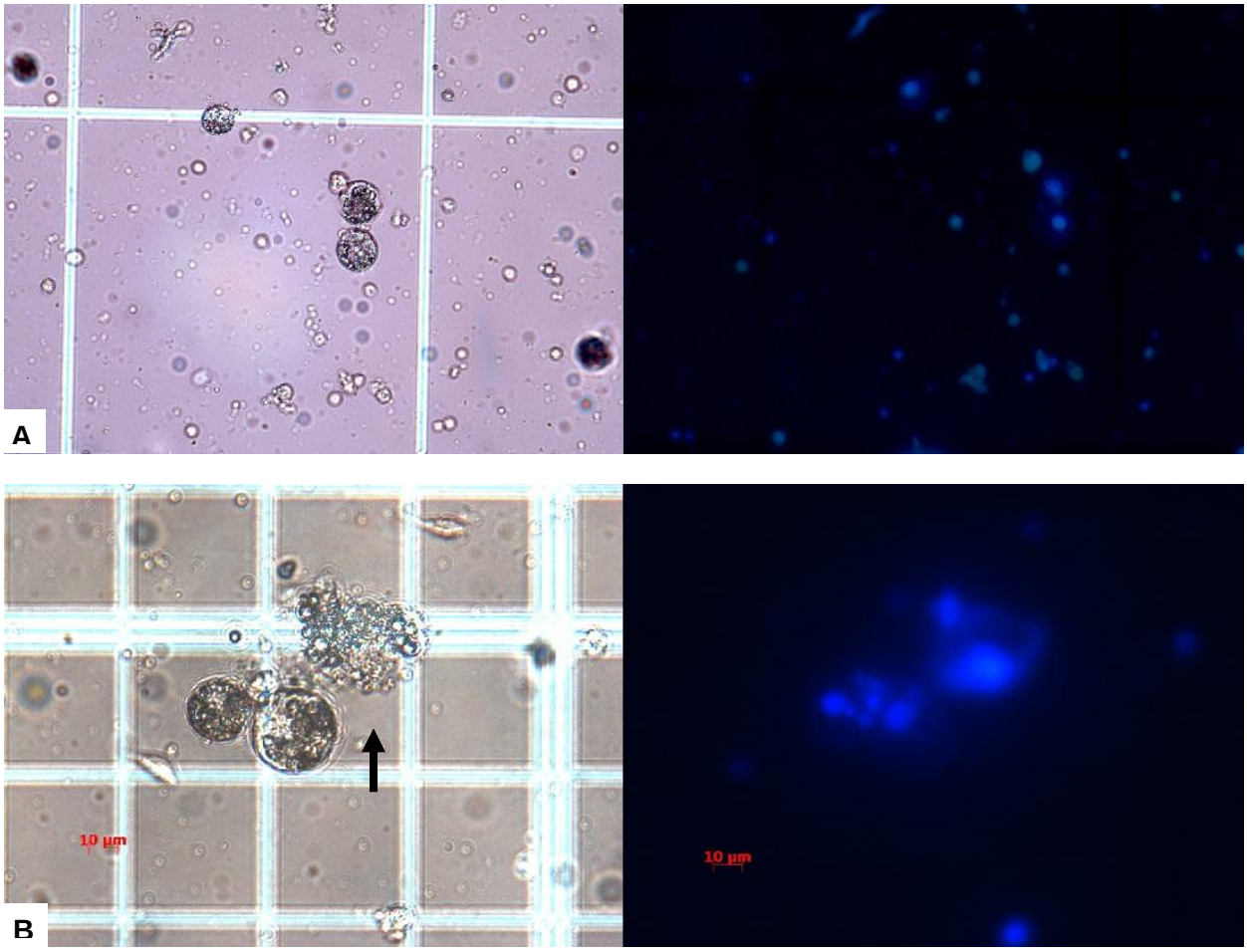

Figure 2. *Dysdercus peruvianus*' hemocytes microaggregation induced by JBU.

Hemolymph was collected from 5th instars in anticoagulant solution and centrifuged for 2 min at 4,400 *g*. Hemocytes were suspended in saline and treated with JBU (200 nM) for 1 hour at room temperature or kept in saline for 1h at room temperature. Both treatments were subsequently incubated with DAPI for 30 min. (A) Control hemocytes, (B) JBU treated hemocytes. Left side – optical microcopy, right side – epifluorescence microscopy. 20x/0.5 magnification. The arrow indicates a microaggregate.

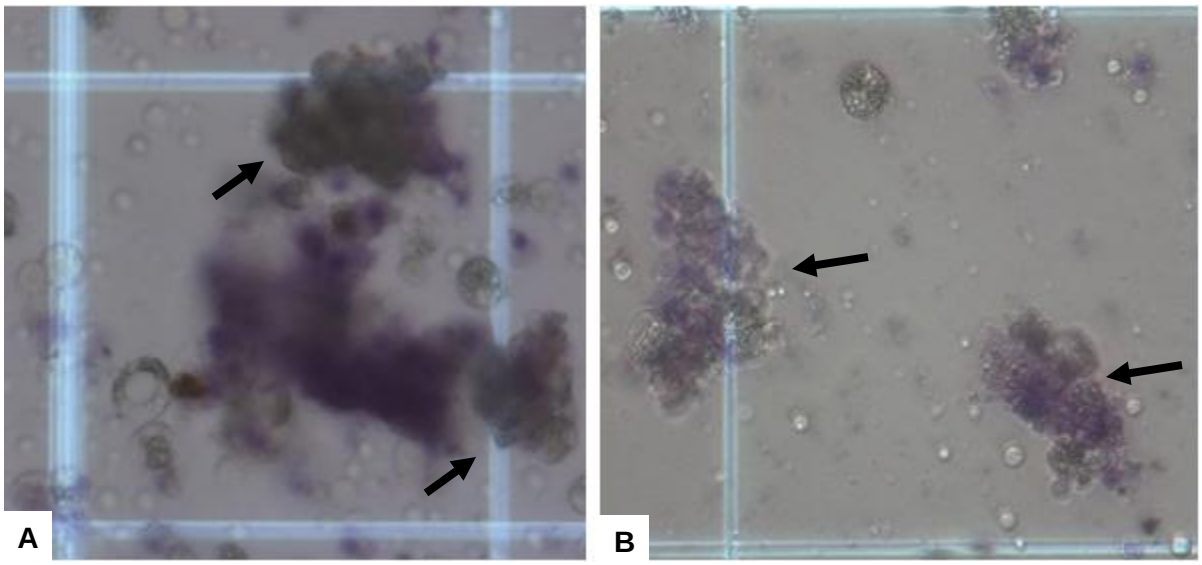

Figure 3. *Dysdercus peruvianus*' hemocytes microaggregation induced by JBU in the absence of Calcium.

Hemolymph was collected from 5th instars in anticoagulant solution and centrifuged for 2 min at 4,400 *g*. Hemocytes were suspended in saline and treated with JBU (200 nM) for 1 hour at room temperature. Trypan blue was added to hemocytes right before microscopy analysis. (A) regular saline, (B) calcium free saline. Regular saline or calcium free saline were used from the first wash to the incubation, respectively. Arrows indicate microaggregates. 20x/0.5 magnification.

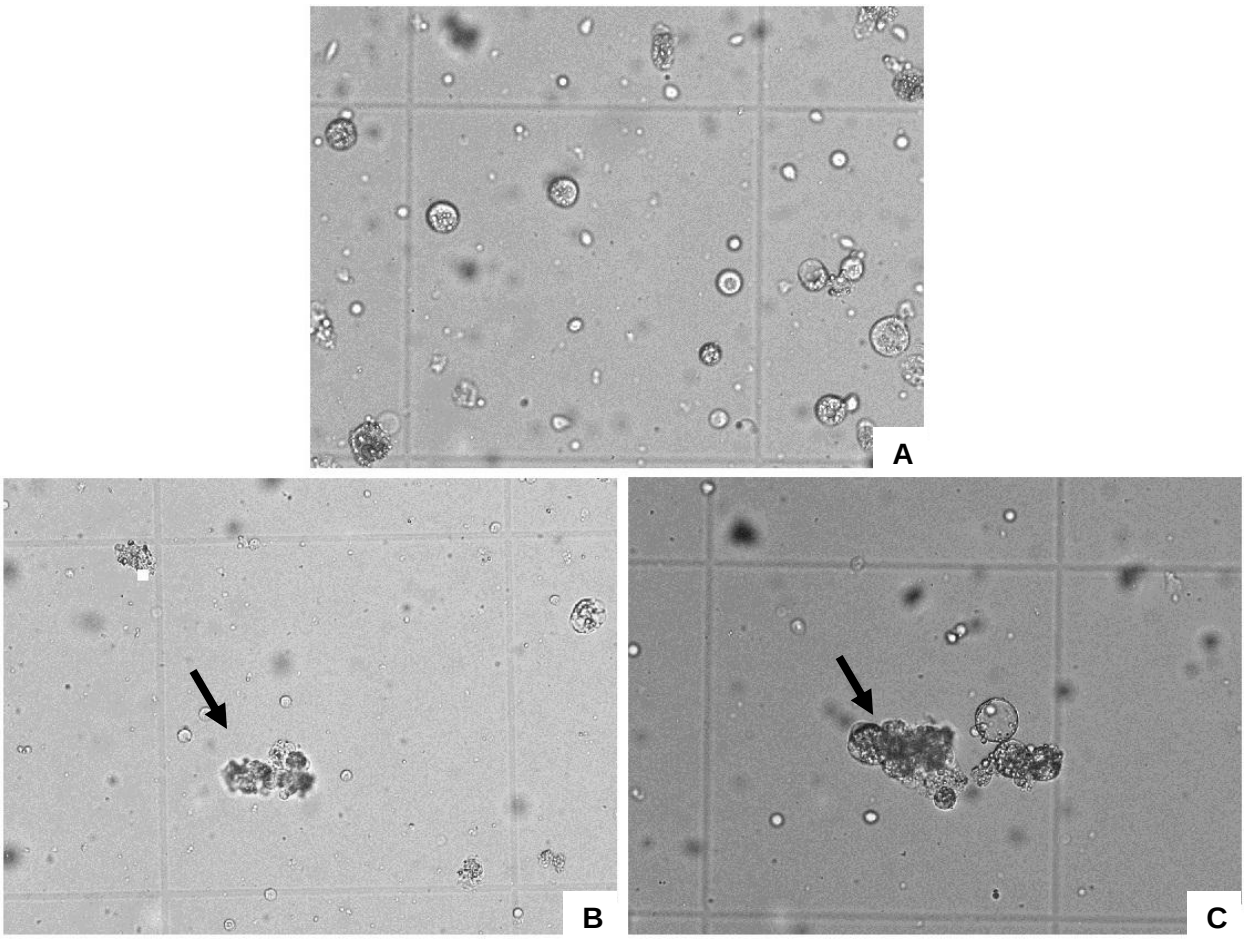

Figure 4. Dexamethasone inhibition of microaggregation induced by JBU in *D. peruvianus*' hemocytes.

Hemolymph was collected from 5th instars in anticoagulant solution and centrifuged for 2 min at 4,400 *g*. Hemocytes were suspended in saline and treated with dexamethasone in different concentrations for 30 min at room temperature. Subsequently, hemocytes were incubated with JBU (200 nM) for 1 hour at room temperature. (A) 0.05 mM dexamethasone, (B) 0.01 mM dexamethasone, (C) 0.005 mM dexamethasone. The arrows indicate the microaggregates. 20x/0.5 magnification.

4. Discussion and Perspectives

The results presented in this work show that hemocytes respond to *Canavalia ensiformis* (jackbean) urease through microaggregation of *D. peruvianus* hemocytes.

Canavalia ensiformis seeds are the natural source of the glucose/mannose specific lectin concanavalin A, well known for its hemagglutinating activity (Carlini & Guimarães, 1991). In this study, highly purified crystalline jackbean urease (JBU) was used to exclude the presence of contaminant concanavalin A. On the other hand, jackbean urease itself was shown to behave as a univalent lectin (thus not able to produce agglutination of cells), binding to sialic acid containing glyconjugates (Follmer *et al.*, 2001). Since insects do not synthesize sialic acid (Friendman, 1985), a possible lectin-like effect of JBU producing the microaggregation of hemocytes was discarded.

In this study, *Canavalia ensiformis* urease was tested for potential actions on immune signaling in the hemipteran model *Dysdercus peruvianus*. I have shown that hemocytes respond to urease through microaggregation of *in vitro* samples of *D. peruvianus* hemocytes. Also in this study, we showed that the glucocorticoid dexamethasone, a PLA2 indirect inhibitor, might have an influence in this reaction. Although dexamethasone has been used as an inhibitor before, nothing has been published about its specificity on eicosanoid pathways within insects. Eicosanoids are known to mediate cellular reaction in insects inoculated with fungi and bacteria (Dean *et al*., 2002; Jurenka *et al*., 1999; Lord *et al*., 2002; Mandato *et al*., 1997; Stanley, 2000; Stanley-Samuelson *et al*., 1991 and 1997) and previous studies showed dexamethasone counteracting the suppression of the immune system by infection within animal models (Garcia *et al*., 2004). Taking into consideration that eicosanoids are molecules that signal inflammatory responses in mammals and signal immune responses in insects, we could deduce that urease is prompting an inflammatory response in *D. peruvianus*. When the experiment was developed with calcium free saline, JBU aggregative activity did not change completely, but reduced the melanized cells and number of aggregates.

In conclusion, JBU was found to directly activate *D. peruvianus* hemocytes in nanomolar doses by inducing an aggregated response. Furthermore, dexamethasone was found to decrease this aggregated response of *D. peruvianus* hemocytes, suggesting eicosanoid metabolites are involved in the reaction. Additional experiments need to be done to elucidate these initial results. Studies with feeding assays and injections of JBU in the hemolymph of *D. peruvianus,* for *in vivo* assays; the use of different eicosanoid synthesis inhibitors, such as

indomethacin and nordihydroguairetic acid; the analysis of hemocytes differentiation; and the establishment of hemocyte cultures, in order to develop different protocols. With this study we were able to show that JBU is acting in hemocyte cellular signaling, opening doors to a novel chapter of this great research topic, the multifaceted insecticidal activity of plant ureases.

5. References

Barcellos, G.B.S., Almeida, L.A., Moreira, R.A., Sousa-Cavada, B., Oliveira, J.T.A., Carlini, C.R. (1993). Canatoxin-cross reactive, concanavalin A-cross reactive and canavalin-cross reactive materials during maturation of *Canavalia brasiliensis* seeds. Planta, 189(3): 397-402.

Barja-Fidalgo,C., Guimaraes,J.A., and Carlini,C.R. (1991a) Canatoxin, a plant protein, induces insulin release from isolated pancreatic islets. *Endocrinology* 128, 675-679.

Barja-Fidalgo,C., Guimaraes,J.A., and Carlini,C.R. (1991b) Lipoxygenase-mediated secretory effect of canatoxin the toxic protein from Canavalia ensiformis seeds. *Toxicon* 29, 453-459.

Becker-Ritt,A.B., Martinelli,A.H., Mitidieri,S., Feder,V., Wassermann,G.E., Santi,L., Vainstein,M.H., Oliveira,J.T., Fiuza,L.M., Pasquali,G., and Carlini,C.R. (2007) Antifungal activity of plant and bacterial ureases. *Toxicon* 50, 971-983.

Barracco,M.A., Rozemary,O., Schlemper JR, B., (1987) The Hemocytes of *Panstrongylus Megistus*. *Mem. Inst, Oswaldo Cruz*. Vol. 82: 431-438.

Buyukguzel,K., Tunaz,H., Putnam,S.M., and Stanley,D. (2002) Prostaglandin biosynthesis by midgut tissue isolated from the tobacco hornworm, Manduca sexta. *Insect Biochem.Mol.Biol.* 32, 435-443.

Carlini,C.R., Guimaraes,J.A., and Ribeiro,J.M. (1985) Platelet release reaction and aggregation induced by canatoxin, a convulsant protein: evidence for the involvement of the platelet lipoxygenase pathway. *Br.J.Pharmacol.* 84, 551-560.

Carlini,C.R. and Guimaraes,J.A. (1991) Plant and microbial toxic proteins as hemilectins: emphasis on canatoxin. *Toxicon* 29, 791-806.

Carlini,C.R. and Polacco,J.C. (2008) Toxic Properties of Urease.*Crop Sci*. 48, 1665-1672.

Carlini,C.R., Oliveira,A.E., Azambuja,P., Xavier-Filho,J., and Wells,M.A. (1997) Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsinlike enzymes. *J.Econ.Entomol.* 90, 340-348.

Costa, S.C., Ribeiro, C., Girard, P.A., Zumbihl, R., Brehelin, M., (2005) Modes of

phagocytosis of gram-positive and gram-negative bacteria by Spodoptera littoralis granular hemocytes. *J. Insect Physiol*. 51, 39–46.

Dean P., Gadsden J.C., Richards E.H., Edwars J.P., Charley A.K., Reynolds S.E. (2002) Modulation by eicosanoid biosynthesis inhibitors of immune responses by the insect *Manduca sexta* to the pathogenic fungus Metarhisium anisopliae. *J Invert. Path.* 79, 93-101

Defferrari,M.S., Demartini,D.R., Marcelino,T.B., Pinto,P.M., and Carlini,C.R. (2011) Insecticidal effect of Canavalia ensiformis major urease on nymphs of the milkweed bug Oncopeltus fasciatus and characterization of digestive peptidases. *Insect Biochem.Mol.Biol.* 41, 388-399.

Dixon,N.E., Gazzola,T.C., Blakeley,R.L., and Zermer,B. (1975) Letter: Jack bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? *J.Am.Chem.Soc.* 97, 4131-4133.

Ferreira-Dasilva,C.T., Gombarovits,M.E., Masuda,H., Oliveira,C.M., and Carlini,C.R. (2000) Proteolytic activation of canatoxin, a plant toxic protein, by insect cathepsin-like enzymes. *Arch.Insect Biochem.Physiol* 44, 162-171.

Follmer, C., Real-Guerra, R., Wasserman, G.E., Olivera-Severo, D., Carlini, C.R. (2004) Jack bean, soybean and Bacillus pasteurii ureases: biological effects unrelated to ureolytic activity. *European Journal of Biochemistry* 271, 1357–1363.

Garcia,E.S., Machado,E.M., and Azambuja,P. (2004) Inhibition of hemocyte microaggregation reactions in Rhodnius prolixus larvae orally infected with Trypanosoma rangeli. *Exp.Parasitol.* 107, 31-38.

Gadelhak,G.G., Pedibhotla,V.K., and Stanley-Samuelson,D.W. (1995) Eicosanoid biosynthesis by hemocytes from the tobacco hornworm, Manduca sexta. *Insect Biochem.Mol.Biol.* 25, 743-749.

Gallo, D. (1988*) Manual de Entomologia Agrńcola*, second ed. CERES, 649 pp.

Ghazaleh,F.A., Araujo,C.F., Barja-Fidalgo,C., and Carlini,C.R. (1992) Canatoxin induces activation on mice peritoneal macrophages. *Braz.J.Med.Biol.Res.* 25, 1033-1035.

Ghazaleh,F.A., Francischetti,I.M., Gombarovits,M.E., and Carlini,C.R. (1997) Stimulation of calcium influx and platelet activation by canatoxin: methoxyverapamil inhibition and downregulation by cGMP. *Arch.Biochem.Biophys.* 339, 362-367.

Giglio, A., Battistella, S., Talarico, F.F., Brandmayr, T.Z., Giulianini, P.G. (2008) Circulating hemocytes from larvae and adults of Carabus (Chaetocarabus) lefebvrei Dejean 1826 (Coleoptera, Carabidae): cell types and their role in phagocytosis after in vivo artificial non-self-challenge. *Micron* 39, 552–558.

Herbert,S.P., Odell,A.F., Ponnambalam,S., and Walker,J.H. (2007) The confluence-dependent interaction of cytosolic phospholipase A2-alpha with annexin A1 regulates endothelial cell prostaglandin E2 generation. *J.Biol.Chem.* 282, 34468-34478.

Jurenka,R.A., Pedibhotla,V.K., and Stanley,D.W. (1999) Prostaglandin production in response to a bacterial infection in true armyworm larvae. *Arch.Insect Biochem.Physiol* 41, 225-232.

Lavine,M.D. and Strand,M.R. (2002) Insect hemocytes and their role in immunity.Insect Biochem. Mol. Biol. 32, 1295–1309.

Lim,L.H. and Pervaiz,S. (2007) Annexin 1: the new face of an old molecule. *FASEB J.* 21, 968-975.

Loher,W., Ganjian,I., Kubo,I., Stanley-Samuelson,D., and Tobe,S.S. (1981) Prostaglandins: Their role in egg-laying of the cricket Teleogryllus commodus. *Proc.Natl.Acad.Sci.U.S.A* 78, 7835-7838.

Lord,J.C., Anderson,S., Stanely,D.W. (2002): Eicosanoids mediate *Manduca sexta* cellular response to the fungal pathogens *Beauveria bassiana*: a role for the lipoxygenase pathway. *Arch. Insect Biocem. Physiol.* 51, 46-54.

Machado,E.M.M., Azambuja,P., Garcia,E.S. (2006) WEB 2086, a platelet-activating factor antagonist, inhibits prophenoloxidase-activating system and hemocyte microaggregation reactions induced by Trypanosoma rangeli infection in Rhodnius prolixus hemolymph. Journal of Insect Physiology 52, 685-692.

Mandato, C.A., Diehl-Jones, W.I., Moore, S.J., Downer, R.G.H. (1997) The effects of eicosanoid biosythesis inhibitors on prophenoloxidase activation, phagocytosis and cell spreading in *Galleria mellonella.* J Insect Physiol.43, 1-8

Meredith, J., Moore, L. and Scudder, G.G.E. (1984) Excretion by ouabain by Malpighian tubules of *Oncopeltus fasciatus*. American Journal of Physiology. 246: R705-R715.

Miller, J.S., and Stanley, D.W. (2001) Eicosanoids mediate microaggregation reactions to bacterial challenge in isolated hemocyte preperations. Journal of Insect Physiology 47, 1409-1417.

Mobley, H.L.T., and Hausinger, R.P. (1989) Microbial ureases: significance, regulation, and molecular characterization. Microbiological Reviews 53, 85-108.

Oliveira, A.E.A., Gomes, V.M., Sales, M.P., Fernandes, K.V.S., Carlini, C.R., Xavier-Filho, J. (1999) The toxicity of jack bean [*Canavalia ensiformis* (L.) Dc] canatoxin to plant pathogenic fungi. Revista Brasileira de Biologia, 59: 59-62.

Piovesan,A.R., Staniscuaski,F., Marco-Salvadori,J., Real-Guerra,R., Defferrari,M.S., and Carlini,C.R. (2008) Stage-specific gut proteinases of the cotton stainer bug Dysdercus peruvianus: role in the release of entomotoxic peptides from Canavalia ensiformis urease. Insect Biochem.Mol.Biol. 38, 1023-1032.

Price, C.D., and Ratcliffe, N.A. (2004) A reappraisal of insect haemocyte classification by the examination of blood from fifteen insect orders. Cell Tissue Res. 147, 537–549.

Ribeiro, C., and Brehelin, M. (2006) Insect hemocytes: what type of cell is that. *J. Insect Physiol*. 52, 417–429.

Staniscuaski,F., Ferreira-Dasilva,C.T., Mulinari,F., Pires-Alves,M., and Carlini,C.R. (2005) Insecticidal effects of canatoxin on the cotton stainer bug Dysdercus peruvianus (Hemiptera: Pyrrhocoridae). Toxicon 45, 753-760.

Staniscuaski,F., TeBrugge, V., Carlini,C.R., and Orchard,I. (2009) Invitro effect of Canavalia ensiformis urease and the derived peptide Jaburetox-2Ec on Rhodnius prolixus Malpighian tubules. J.Insect Physiol 55, 255-263.

Staniscuaski,F., TeBrugge, V., Carlini,C.R., and Orchard,I. (2010) Jack bean urease alters serotonin-induced effects on Rhodnius prolixus anterior midgut. J.Insect Physiol 56, 1078-1086.

Stanley,D. (2006) Prostaglandins and other eicosanoids in insects: biological significance. Annu.Rev.Entomol. 51, 25-44.

Stanley,D., Miller,J., and Tunaz,H. (2009) Eicosanoid actions in insect immunity. J.Innate.Immun. 1, 282-290.

Stanley,D.W. (2000) Eicosanoids in Invertebrate Signal Transduction Systems. Princeton, Princeton University Press.

Stanley-Samuelson,D.W. and Ogg,C.L. (1994) Prostaglandin biosynthesis by fat body from the tobacco hornworm, *Manduca sexta*. Insect Biochem.Mol.Biol. 24, 481-491.

Strand, MR. (2008) Insect hemocytes and their role in immunity; in Beckage NE (ed): Insect Immunology. Amsterdam, Elsevier, 25–47.

Sumner, J.B. (1926). The isolation and crystallization of the enzyme urease. Journal of Biological Chemistry, 69: 435-441.

Wassermann,G.E., Olivera-Severo,D., Uberti,A.F., and Carlini,C.R. (2010) Helicobacter pylori urease activates blood platelets through a lipoxygenase-mediated pathway. *J.Cell Mol.Med.* 14, 2025-2034.